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Transcriptional Network of Multiple Capsule and Melanin Genes Governed by the *Cryptococcus neoformans* Cyclic AMP Cascade

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Cryptococcus neoformans **is an opportunistic human fungal pathogen that elaborates several virulence attributes, including a polysaccharide capsule and melanin pigments. A conserved Gα protein/cyclic AMP (cAMP) pathway controls melanin and capsule production. To identify targets of this pathway, we used an expression profiling approach to define genes that are transcriptionally regulated by the Gα protein Gpa1. This approach revealed that Gpa1 transcriptionally regulates multiple genes involved in capsule assembly and identified two additional genes with a marked dependence on Gpa1 for transcription. The first is the *LAC1* gene, encoding the laccase enzyme that catalyzes a rate-limiting step in diphenol oxidation and melanin production. The second gene identified (*LAC2*) is adjacent to the *LAC1* gene and encodes a second laccase that shares 75% nucleotide identity with *LAC1*. Similar to the *LAC1* gene, *LAC2* is induced in response to glucose deprivation. However, *LAC2* basal transcript levels are much lower than those for *LAC1*. Accordingly, a *lac2* mutation results in only a modest delay in melanin formation. *LAC2* overexpression suppresses the melanin defects of *gpa1* and *lac1* mutants and partially restores virulence of these strains. These studies provide mechanistic insights into the regulation of capsule and melanin production by the *C. neoformans* cAMP pathway and demonstrate that multiple laccases contribute to *C. neoformans* melanin production and pathogenesis.**

Cryptococcus neoformans is a human fungal pathogen that primarily infects immunocompromised hosts. In order to establish an infection, pathogenic microorganisms such as *C. neoformans* must sense host-specific signals and respond with specific adaptive cellular responses that allow their survival in this hostile environment. Accordingly, *C. neoformans* requires the induction of several factors to be fully virulent. These include an antiphagocytic polysaccharide capsule (19, 27) and production of antioxidant melanin pigments (26).

The importance of capsule and melanin in *C. neoformans* infections has been studied extensively. The expression of each is induced in response to environmental signals encountered in the host during an infection. For example, capsule formation is induced by severe iron deprivation (55), mammalian physiologic concentrations of CO_2/HCO_3^- (19), or serum (61). Melanin production requires glucose deprivation and the presence of diphenolic substrates, such as catecholamines (39).

Melanins represent a group of dark pigments present in a variety of fungal species. Plant fungal pathogens, such as *Magnaporthe grisea*, produce melanin in specialized structures known as appresoria to generate the physical pressure required to penetrate plant cells and establish an infection (32). Melanin formation in human fungal pathogens has also been linked to pathogenicity. For example, melanin-deficient mutants of the dematiaceous mold *Wangiella dermatitidis* are avirulent in animal models compared to wild-type controls (15).

In *Cryptococcus neoformans*, melanin-deficient mutant strains are also attenuated for virulence (26). Therefore, several investigators have focused on defining the biochemical steps involved in melanin biosynthesis and the potential roles for melanin in the pathogenesis of cryptococcal infections. The synthesis of melanin in *C. neoformans* involves the oxidation of phenolic substrates into quinones, which then polymerize nonenzymatically into pigmented products (59). The *LAC1* gene encoding an enzyme catalyzing the rate-limiting oxidation step in melanin biosynthesis was cloned, and its product was identified as a laccase (59). Laccases are known to have diphenol oxidase activity and utilize a wide array of substrates, oxidizing polyaminobenzenes and mono- or polyphenolic compounds. Diphenol oxidase activity is found in strains producing melanin and is absent in melanin-deficient strains of *C. neoformans* (59). Strains with a *lac1* mutation are attenuated for virulence in animal models but are eventually able to establish a lethal infection (46). Also, *lac1* mutant strains can produce melanin after prolonged incubation on melanin-inducing media. Therefore, Lac1 plays a central role in catecholamine oxidation and melanin production, but other proteins or enzymes may function together with this enzyme in melanin biosynthesis.

Studies with *C. neoformans* reveal that a conserved Gα protein-cyclic AMP (cAMP) pathway regulates the induction of capsule and melanin in response to environmental stimuli detected in the host. The Gpa1-cAMP signaling cascade is conserved in yeast and mammals; however, the functions served by this pathway differ markedly between organisms. In the nonpathogenic fungus *Saccharomyces cerevisiae*, for example, this cascade plays central roles in filamentation, sporulation, and stress survival (4, 41, 45). In a similar manner, the human fungal pathogen *C. neoformans* utilizes cAMP signaling to reg-

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ulate morphological transitions, but it has also coopted this cascade for the regulation of cellular determinants involved in virulence. The conserved components of a cAMP-signaling cascade have been characterized and involve genes encoding the Gα protein (Gpa1), adenylyl cyclase (Cac1), and protein kinase A catalytic subunit (Pka1). Mutant strains lacking these components do not increase capsule or melanin production in response to normal inducing conditions (2, 3, 17). However, little is known about the molecular mechanisms employed by cAMP to induce the expression of these virulence determinants. Here, we used a genomic microarray to identify genes whose expression is dependent upon Gpa1 to define downstream targets of this signal transduction pathway. Using this approach, we demonstrate that the cAMP pathway controls capsule and melanin production by regulating the expression of multiple genes at the transcriptional level. Additionally, we identify and characterize a second gene encoding a laccase homolog involved in melanin production in *C. neoformans*.

MATERIALS AND METHODS

Strains and media. The strains used are listed in Table 1. All strains in this study were derivatives of the serotype A wild-type strain H99 (42), with the exception of those in the first microarray experiment, which was performed using the wild-type strain JEC21 and the *gpa1* mutant strain BAC21 (54).

Standard yeast media were used as described previously (49). Niger seed extract and agar were also used (25). As indicated, media were supplemented with 10 mM epinephrine, 0.5 mM dopamine, or 100 μM 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS).

RNA preparation. The isogenic *GPA1* wild-type and *gpa1* mutant strains were grown for 24 h at 30°C in synthetic complete (SC) medium with 2% glucose. Strains were pelleted, resuspended in SC medium with 2% glucose, and incubated for 3 h at 30°C. The cells were pelleted and resuspended in either SC medium with 2% glucose, SC with 0% glucose, or SLAD (synthetic low ammonium dextrose) medium. Aliquots were collected by centrifugation at 0, 1, and 2 h and flash frozen on dry ice. Total RNA was extracted from lyophilized cells by using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, Calif.).

cDNA synthesis and labeling. Fluorescently labeled cDNA was made by incorporating amino-allyl dUTP during reverse transcription of 10 μg of total RNA. Cy3 or Cy5 fluorescent dyes (Amersham, Piscataway, N.J.) were coupled to the amino-allyl group as previously described (14). We generated a reference sample by pooling an equal amount of RNA from each time point from both strains, converted to cDNA and labeled with Cy3. RNA from each time point was individually labeled with Cy5 and competitively hybridized against the reference sample.

Microarray hybridization and analysis. The genomic DNA microarray construction was described in detail previously (23). Briefly, 6,144 PCR products were amplified from a 1.6- to 3.2-kb genomic library made with strain H99 genomic DNA and were printed on polylysine-coated glass slides. Slides were prehybridized at 42°C with 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate, and 1% bovine serum albumin, and hybridizations were performed at 42°C with 1× hybridization buffer (50% formamide, 5× SSC, 0.1% sodium dodecyl sulfate). Arrays were scanned with a GenePix 4000B scanner (Axon Instruments, Foster City, Calif.) and analyzed by using GenePix Pro v4.0. Further data analysis was performed with CryptoArray, a Microsoft Excel macro for normalizing and formatting data. The smaller microarray was constructed in a similar manner by printing PCR products from 111 genes from strain JEC21 on polylysine-coated slides and hybridizing, as described above, with fluorescently labeled cDNA from the wild-type strain JEC21 and the *gpa1* mutant strain BAC21.

Creation of *lac2*, *lac1*, and *lac1 lac2* mutants. To create the *lac2*::*neo* disruption allele, the PCR overlap extension method was used as previously described (11). The left fragment was amplified from H99 genomic DNA, using primers AAO304 and AAO306. The right fragment was amplified from H99 genomic DNA, using primers AAO307 and AAO309. The central fragment, containing the neomycin-resistance selectable marker, was amplified from plasmid pJAF1, using primers AAO305 and AAO308. The three PCR amplicons were used collectively as the template for a final PCR with primers AAO304 and AAO309 to generate the 4.0-kb *lac2*::*neo* disruption construct. The *lac1*::*URA5* and *lac1*::*nat* disruption constructs were similarly created by the PCR overlap method. The constructs were designed such that the *neo* and the *URA5* genes completely replaced the *LAC2* and *LAC1* genes, respectively, from start to stop codon.

TABLE 1. Strain list

Strain	Genotype	Reference or source
H99	*MAT*α wild-type	42
AAC51	*MAT*α *ura5*	3
AAC1	*MAT*α *ade2 gpa1*::*ADE2*	2
AAC4	*MAT*α *ade2 gpa1*::*ADE2 ura5*	This study
RPC26	*MAT*α *lac2*::*neo*	This study
RPC27		
RPC28		
QGC4	*MAT*α *lac2*::*neo pGPD-LAC2*	This study
QGC5		
QGC6		
MDJ12	*MAT*α *lac1*::*nat1*	This study
MDJ13		
MDC16	*MAT*α *ura5 lac1*::*URA5*	This study
MDC17		
RPC29	*MAT*α *ura5 lac1*::*URA5 lac2*::*neo*	This study
RPC30		
QGC8	*MAT*α *lac1*::*nat1 lac2*::*neo*	This study
QGC9		
RPC18	*MAT*α *ade2 gpa1*::*ADE2 pGPD-LAC2*	This study
RPC19		
RPC20		
RPC21	*MAT*α *pGPD-LAC2*	This study
RPC22		
RPC23		
QGC1	*MAT*α *ura5 lac1*::*URA5 pGPD-LAC2*	This study
QGC2		
QGC3		

The disruption constructs were used to biolistically transform *C. neoformans* strains as previously described for gene disruption (52). The *lac2*::*neo* and *lac1*::*nat* constructs were transformed into strain H99 to generate the *lac2* mutant strains RPC26, RPC27, and RPC28 and the *lac1* mutant strains MDJ12 and MDJ13. The *lac2*::*neo* construct was transformed into the *lac1* mutant strain MDJ12 to create the *lac1 lac2* double-mutant strain QGC9.

To screen for the *lac2* mutation, genomic DNA from each transformant was isolated and used in a PCR with the *LAC2*-specific primer AAO303 and the *neo*-specific primer AAO247 (Table 2). Because the recognition sequence of primer AAO303 is outside of the disruption construct, only those transformants that have the disruption construct at the site of the endogenous *LAC2* gene will amplify a 1.2-kb product. Three *lac2*::*neo* mutant strains and two *lac1 lac2* double-mutant strains were identified in this manner. Each strain had phenotypes identical to those of the other genotypically identical mutant strains.

Southern hybridization was performed to confirm gene disruption with genomic DNA digested with PstI and XhoI and using as a probe a 3.1-kb fragment of the *LAC2* gene created using primers AAO314 and AAO315. We observed the expected 2.3- and 1.7-kb band in both *lac2* mutants as well as a 4.3-kb band corresponding to cross-hybridization at the *LAC1* locus. This signal is less intense than the wild-type *LAC2* signal at 4.1 kb and is not present in the *lac1 lac2* double mutant, in which the coding region of *LAC1* has been replaced with *URA5*.

Overexpression and reconstitution of the *LAC2* gene. Plasmid pRCD83 contains the sequence of the constitutively active promoter of the *GPD* gene and *URA5* selectable marker (36, 58). We cloned the coding sequence of the *LAC2* gene into this vector under control of the *GPD* promoter. The *LAC2* gene was amplified from serotype A genomic DNA, using primers AAO314 and AAO315, which contain KpnI and BamHI restriction endonuclease sites. The PCR fragment was ligated into a KpnI- and BamHI-cut pRCD83 vector to create plasmid pRPW1 (*pGPD-LAC2*). The overexpression construct was biolistically transformed into AAC51 (*ura5*) and AAC4 (*gpa1 ura5*). To introduce this plasmid into the *lac1* mutant and the *lac2* mutant, the dominant selectable marker encoding nourseothricin resistance was subcloned into pRPW1. The 1.8-kb nourseothricin-resistance gene (31) was excised from plasmid pCH233 by using SpeI and XbaI and cloned into an XbaI-cut pRPW1. This new plasmid, pQDG1, was biolistically transformed into RPC32, creating strains QGC4, QGC5, and QGC6. Stable genomic integration of the constructs was documented by repeated culture of the transformants on a nonselective medium, followed by

TABLE 2. Primer list

Primer	Purpose[a]	Sequence (5′-3′)	Note	Annealing position
AAO304	*lac2*::*neo* construct	GGTATCTGACGGCATTAGAAGG	419 bp upstream of *LAC2* start site	*LAC2* (−419)–(−398)
AAO306	PCR overlap	CGTGTTAATACAGATAAACCAAGGGT TAGCCTCTATCACAGGTCC	*neo* marker sequence underlined	*LAC2* 576–596
AAO307	PCR overlap	GCTCACATCCTCGCAGCAAGGGAGAA TGCCTGGACATCTCATGC	*neo* marker sequence underlined	*LAC2* 2743–2765
AAO309	*lac2*::*neo* construct	CTGCTCCTCTAGATCACTAACGTCAGG	Amplifies out of *neo* marker	
AAO305	PCR overlap	GACCTGTGATAGAGGCTAACCCTTGG TTTATCTGTATTAACACGG	*neo* marker sequence underlined	*LAC2* 576–596
AAO308	PCR overlap	GCATGAGATGTCCAGGCATTCTCCCT TGCTGCGAGGATGTGAGC	*neo* marker sequence underlined	*LAC2* 2743–2765
AAO303	*lac2*Δ screen	ATCAGCTATATCACCTGTCAAGGC	Upstream of *LAC2* locus	*LAC2* (−598)–(−575)
AAO247	*neo* marker screen	CGTTGAATCCTCAGGATCTTCATGGC	Amplifies out of *neo* marker	
AAO314	*LAC2* probe	CCGGATCCTCTGACACATTCACAACA ATGG	BamHI sequence underlined	*LAC2* (−20)–4
AAO315	*LAC2* probe	GCGGTACCGGACGAAGGTAATAGCA GAGAGTCAGG	KpnI sequence underlined	*LAC2* 3062–3088
AAO463	*LAC2* cDNA	ACATCATATCTCTATCTTCAAGG	Primer sequence specific to *LAC2*	*LAC2* (−46)–(−24)
AAO534	*LAC2* cDNA	CAGGCCATTGAATCTTTTTG	Primer sequence specific to *LAC2*	*LAC2* 2546–2565
AAO570	*LAC2* qRT-PCR	TGTATGGCGCAAGGGGTTACT	Primer sequence specific to *LAC2*	*LAC2* 1657–1677
AAO571	*LAC2* qRT-PCR	AGAACACGACTCTCCAAAGC	Primer sequence specific to *LAC2*	*LAC2* 1948–1967
AAO301	*GPD* qRT-PCR	AGTATGACTCCACACATGGTCG	Primer spans intron V of *GPD*	*GPD* 405–415; 471–481
AAO302	*GPD* qRT-PCR	AGACAAACATCGGAGCATCAGC	Primer spans intron VI of *GPD*	*GPD* 693–704; 764–773

[a] qRT-PCR, quantitative reverse transcriptase PCR.

culturing of the strains on either synthetic medium lacking uracil (strains transformed with pRPW1) or yeast-peptone-dextrose (YPD) medium containing nourseothricin (strains transformed with pQDG1). Overexpression of the *LAC2* gene in a wild-type strain, the *lac1* mutant, the *lac2* mutant, the *lac1 lac2* mutant, and the *gpa1* mutant was confirmed by Northern blot analysis.

Sequencing of *LAC2* cDNA. We amplified *LAC2* cDNA from a cDNA library from strain H99 using primers designed to specifically amplify the *LAC2* sequence over that of the highly similar *LAC1* gene: primer AAO463 (begins at position −46 relative to the predicted start codon) and primer AAO534. The cDNA sequence confirmed predicted intron-exon borders.

Quantification of *LAC2* transcript. *C. neoformans* strains were incubated in YPD medium with 2% glucose to mid-log phase, pelleted, and resuspended in yeast nitrogen base medium (YNB) without added glucose (or other inducing conditions as listed in Results) for 1 h. The cells were pelleted and flash frozen on dry ice, and total RNA was isolated from these isolates by using TRIzol as previously described (3). The RNA was treated with RNase-free DNase, and cDNA was synthesized using oligo-dT primers from the SuperScript First Strand Synthesis RT kit (Invitrogen). The resulting cDNA was used as a template for quantitative real-time PCR with iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's specifications. The iCycler iQ Multicolor real-time detection system was used as the fluorescence detector with the following PCR conditions: an initial denaturing cycle of 95°C for 3 min, 40 cycles of denaturation at 95°C for 20 s, and annealing and extension at 53°C for 45 s. These cycles were followed by a standard melt curve from 53 to 93°C with fluorescent monitoring each 0.5°C. These data confirmed the amplification of a single product for each primer pair and the lack of primer dimerization. Reactions were performed in triplicate, and the data were expressed as an average cycle threshold (C_T) value, plus or minus standard error. The *LAC2* primers used in this reaction (AAO570 and AAO571) amplify a 208-bp amplicon near the 3′ end of the posttranscriptionally modified cDNA. Standard PCRs were run with fivefold dilutions of the cDNA template to determine the optimal amount of template and optimal annealing temperature for the experimental and reference reactions, using a 500 nM concentration of each primer.

A validation curve was also calculated for each cDNA sample to provide an index of the template quality and quantity for each sample. The mean expression level for each gene in each sample was regressed against the overall mean of all samples. The slope provides an estimate of the degree to which the gene is efficiently amplified in the reaction, and applying this r^2 value to the statistical evaluation for expression accounts for unpredictable variation between samples.

LAC2 amplification for each strain and condition was normalized against the constitutively expressed *GPD* gene. Degree of induction was calculated relative to induction for the wild-type strain H99, using the Bio-Rad iCycler software system, which utilizes the comparative C_T statistical methods as previously described (54).

Northern analysis. Total RNA was prepared as described above. Fifteen micrograms of total RNA was analyzed for each sample. Gel electrophoresis, RNA transfer, hybridization, and autoradiography were performed as described previously (47). The probes used for Northern analysis included the entire coding sequences of *GPA1* (53), *CAS1* (22), *CAS2* (34), *SMG1* (unpublished data), and *ACT1* (10), amplified by PCR from a JEC21 cDNA library. To minimize potential cross-hybridization between the highly related *LAC1* and *LAC2* genes, we specifically chose as probes the extreme 3′ region of the coding sequences, since this includes the most dissimilar regions of the *LAC1* and *LAC2* genes. The *LAC1* probe corresponded to nucleotides 1392 to 1872 of the *LAC1* cDNA sequence, and the *LAC2* probe corresponded to nucleotides 1019 to 1715 of the *LAC2* cDNA sequence. Although we cannot exclude some degree of cross-hybridization of the *LAC1* probe with the *LAC2* signal, the absence of detectable *LAC2* signal on several Northern blots (see Fig. 3C; also unpublished data) likely minimizes the effect of this possibility. The DNA for probes was labeled using a Random Primed DNA labeling kit (Boehringer Mannheim) and ^{32}P-dCTP (Amersham).

Southern analysis. Genomic DNA was isolated from strains by using previously described techniques (44). Restriction digestion, gel electrophoresis, DNA transfer, prehybridization, hybridization, and autoradiography were performed as described previously (47).

PCR. All PCRs were carried out with a Techne Genius thermocycler with 50 ng of template DNA, 100 ng of each oligonucleotide primer, and standard reagents from a TaKaRa kit (Takara Shuzo Co.). The PCR conditions were 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min for each kilobase amplified in the reaction.

Virulence experiments. In the murine inhalation model of systemic cryptococcosis, A/Jcr mice were intranasally inoculated with 10^5 cells as previously described (9). Groups of 10 mice were inoculated with each strain in the study and observed twice daily for signs of infection. The statistical significance in the difference between each strain's resulting survival rate was evaluated with the Mann-Whitney statistical model.

In this model, mice develop meningoencephalitis after inhalation of *C. neoformans*, a course that mimics the natural history of human infection with this organism. Signs consistent with cryptococcosis in this experimental model included lethargy, ruffled fur, and inability to maintain daily care. Moribund mice were sacrificed prior to death, and all studies were performed in compliance with institutional guidelines for animal experimentation. All surviving mice were sacrificed at 80 days after infection.

RESULTS

Transcriptional profiles of the wild-type and *gpa1* mutant strains. Previous studies demonstrated that the Gpa1-adenylyl cyclase signaling cascade in *C. neoformans* controls the expression of two inducible virulence factors, capsule and melanin. To identify genes whose transcription is dependent on the Gα

TABLE 3. Microarray comparison of gene transcript levels in the wild-type strain versus the *gpa1* mutant strain

Gene	Encoded protein	*GPA1*/*gpa1*[a]	Reference
GPA1	Gα protein	8.8	2, 53
CAS8	Capsule-associated gene	6.1	G. Janbon, unpublished data
CAS2	UDP-xylose synthase	4.9	34
CAP10	Capsule-associated gene	4.5	7
CAS1	*O*-acetyl transferase	3.7	22
CAT2	Catalase	3.7	Unpublished
SMG1	Suppressor of *gpa1* mutant phenotypes	3.6	Unpublished
CAS7	Capsule-associated gene	3.4	G. Janbon, unpublished
CAP59	Capsule-associated gene	3.2	5
CAP64	Capsule-associated gene	3.1	8
RLM1	Serum response-like factor protein homolog	3.1	Unpublished data
THR4	Threonine synthase	3.1	Unpublished data
CAS4	Capsule-associated gene	2.9	G. Janbon, unpublished
CAS31	Capsule-associated gene	2.8	33
GPA2	Gα protein	2.8	Unpublished

[a] *GPA1*/*gpa1* indicates the relative transcript level in the *GPA1* wild-type strain compared to the *gpa1* mutant strain as assessed by microarray analysis.

protein Gpa1, we employed genome microarrays to compare the transcriptional profiles of wild-type and *gpa1* mutant strains.

A DNA microarray was created by printing PCR-amplified cDNAs from 111 known *C. neoformans* genes onto glass slides. Several genes are present multiple times, and all cDNAs were printed in duplicate, resulting in a microarray that contains 260 elements. This slide was simultaneously hybridized with cDNA from a wild-type strain labeled with the Cy3 (green) fluorophore and cDNA from a *gpa1* mutant strain labeled with Cy5 (red). cDNA from each strain was synthesized from total RNA extracted from cells after 1 h of glucose starvation. Glucose starvation was predicted to induce some Gpa1-regulated genes because this condition is required for *C. neoformans* melanin production.

To define genes whose transcription is regulated by Gpa1, we calculated the relative fluorescence intensities for each microarray spot when probed with these different fluorophore-labeled cDNA pools. The expression of 15 genes was increased at least 2.5-fold in the wild-type strain compared to that in the *gpa1* mutant. The gene with the greatest difference in expression was the *GPA1* gene itself, providing an important internal control for this experiment.

Of the other 14 genes, nine are known or presumed to function in capsule synthesis or assembly (Table 3). The *CAS1* gene encodes an *O*-acetyltransferase, and *CAS2* encodes a UDP-xylose synthase, both of which are required for the assembly of glucoronoxylomannan, the primary component of the cryptococcal capsule (22, 34). The *CAP10*, *CAP59*, and *CAP64* genes encode proteins of unknown function, but each of these genes is required for *C. neoformans* capsule formation and virulence (5, 7, 8). The other *CAS* genes were identified in prior genetic screens, and mutations in these genes are associated with capsule defects (G. Janbon, personal communication).

To confirm these findings, we performed Northern analysis of total RNA isolated from the wild-type and *gpa1* mutant strains after 1 h of glucose starvation, using several genes from the microarray experiment as probes. In contrast to the case with the wild type, there was no *GPA1* signal detected in RNA from the *gpa1* mutant strain. As predicted by the microarray analysis data, expression of the *CAS1*, *CAS8*, and *SMG1* genes was increased in the *GPA1* wild-type strain compared to that in the *gpa1* mutant (Fig. 1). The *SMG1* gene was identified in an independent genetic screen for suppressors of *gpa1* mutant phenotypes (unpublished results), and it is therefore reasonable that Gpa1 may regulate the expression of this gene.

Identification of a second laccase gene (*LAC2*) in *C. neoformans* using a genome microarray. As a complementary approach, we used a genomic DNA microarray containing more than 6,000 elements to assess Gpa1-regulated expression of potentially unknown genes. This microarray was produced by using PCR products amplified from a 1.6- to 3.2-kb insert genomic library constructed by using strain H99 genomic DNA. The utility of this genomic DNA microarray for assessing gene expression has previously been demonstrated in a study of temperature-regulated gene expression (23). cDNA samples were prepared in the manner described above and hybridized to the genomic microarray.

Using the larger microarray, we identified six genomic fragments that demonstrated a fivefold or greater transcription level in the *GPA1* wild-type strain compared to the *gpa1* mutant strain (Fig. 2A). The genomic fragment s0011P0069Z_A11 demonstrated a 28.8-fold transcriptional dependence on Gpa1. A nucleotide BLAST search of this fragment against the H99 genome database revealed that this genomic fragment is included within the *LAC1* locus (Fig. 2B). Northern blot analysis confirmed that the *LAC1* gene is transcriptionally regulated by Gpa1. The *LAC1* transcript is not detectable in *gpa1* mutants after 1 h of glucose starvation, but it can be detected at similar levels in a wild-type strain and in a *gpa1*+*GPA1* reconstituted strain (*gpa1* mutant transformed with a wild-type *GPA1* gene) (2) incubated under identical conditions (Fig. 2C). This observation is consistent with the melanin-deficient phenotype of the *gpa1* mutant strain and provides insight into the molecular mechanism by which the Gpa1 protein controls melanin production.

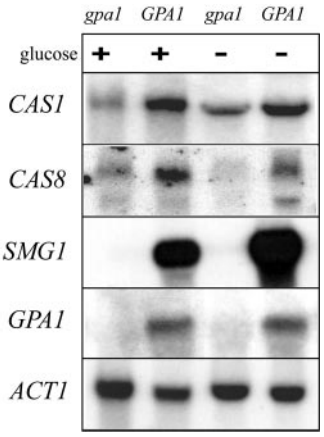
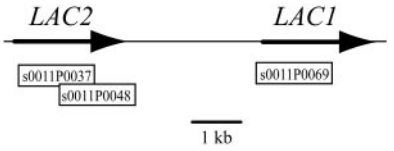


FIG. 1. Northern analysis confirms the *gpa1* mutant microarray results. The wild-type (*GPA1*) and *gpa1* mutant strains were incubated to mid-logarithmic phase in YPD and exposed for 1 h to glucose-rich (+) or glucose-poor (−) conditions. Total RNA was extracted from these strains and used for Northern analysis with the *CAS1*, *CAS8*, *SMG1*, and *GPA1* genes as probes, with the *ACT1* (actin) gene as a loading control.

A

Sequence	*GPA1/gpa1*	
s0011P0069Z_A11	28.8	*LAC1*
s0011P0048Z_A9	10.0	*LAC2*
s0011P0050Z_E2	7.2	
s0011P0033Z_A7	7.1	
s0011P0033Z_B5	5.8	
s0011P0037Z_E9	5.3	*LAC2*

B



C

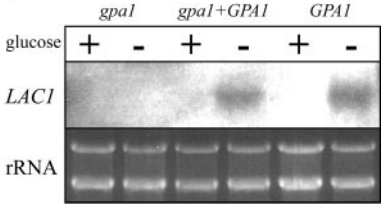


FIG. 2. *LAC1* and *LAC2* transcript levels are regulated by Gpa1. (A) Gene microarray analysis identified six genomic sequences of the serotype A strain H99 that exhibit an increased ratio of cDNA hybridization in the wild-type strain compared to the *gpa1* mutant (*GPA1*/*gpa1*), suggesting that a gene present in these sequences is differentially expressed in these two strains. (B) One of these sequences (s0011P0069Z_A11) localized to the region of the *LAC1* gene, and two sequences (s0011P0048Z_A9 and s0011P0037Z_E9) localized 4 to 5 kb upstream of *LAC1*, in a region containing a second putative laccase gene, *LAC2*. (C) To confirm the microarray results, Northern analysis using the *LAC1* gene as a probe was performed with total RNA from the wild type (H99), the *gpa1* mutant (AAC1), and the *gpa1*+*GPA1* reconstituted strain (AAC3) after 1 h of incubation under glucose-rich (+) or glucose-poor (−) conditions. The rRNA signal in the ethidium bromide-stained RNA gel is shown to demonstrate equal RNA loading.

Two other DNA fragments demonstrated a marked transcriptional dependence on Gpa1 and were transcribed at 10.0- and 5.3-fold-higher levels in the wild-type than in the *gpa1* mutant strain, respectively. Both fragments showed perfect nucleotide identity to a region approximately 5 kb upstream of the *LAC1* start codon (Fig. 2A and B). Interestingly, these two fragments (s0011P0048Z_A9 and s0011P0037Z_E9) also demonstrated 75 and 76% nucleotide identity, respectively, with the *LAC1* locus. Further analysis of this region of the *C. neoformans* genome revealed the presence of a putative gene encoding a laccase homolog, distinct from *LAC1*, which we designated *LAC2*.

The *LAC2* gene consists of 2,690 nucleotides from the start to termination codons, contains 13 introns, and encodes a predicted protein of 596 amino acids. The *C. neoformans LAC2* cDNA sequence shares 85% nucleotide identity with *LAC1* in the first 1,063 nucleotides and 75% identity from nucleotides 1252 to 1788. *LAC2* also shares significant homology with other fungal laccases and enzymes involved in pigment formation: 30% amino acid identity with the laccase 2 gene of *Botryotinia fuckeliana* (48) and 29% identity with a *LAC1* precursor gene of *Agaricus bisporus* (43) and an *Neurospora crassa* conidial pigment biosynthesis protein (GenBank accession no. CAD 70788). Laccase proteins contain several conserved motifs, including substrate-binding and copper-binding domains. These laccase signature motifs, L1 to L4 (24), are all conserved in the predicted Lac2 protein.

Lac2 is transcriptionally repressed by glucose. To define the conditions that regulate the expression of the *LAC2* gene, we used quantitative real-time PCR. This method was chosen for two reasons. First, we wished to avoid potential cross-hybridization with the highly related *LAC1* gene in Northern blots. Also, initial Northern hybridizations that used the most dissimilar regions of *LAC1* and *LAC2* as probes suggested that the *LAC2* gene is transcribed at low levels under several growth conditions (data not shown).

To assess *LAC2* transcription by real-time PCR, the wild-type strain H99 was incubated to mid-logarithmic phase in YPD medium and shifted to one of several inducing conditions: YPD (0.01% glucose), YPD plus 100 μM copper sulfate, YPD plus 0.5 mM hydrogen peroxide, YPD plus 10 mM paraquat, YNB (pH 7) (with or without 0.5 mM $NaNO_2$), and YNB (pH 4) (with or without 0.5 mM $NaNO_2$). Acidic aqueous solutions containing $NaNO_2$ (YNB [pH 4] plus $NaNO_2$) provide nitric oxide-inducing conditions (1). Total RNA was isolated from the cell pellets and treated with DNase I, and first-strand cDNA was synthesized with reverse transcriptase. This cDNA was used as the template for quantitative real-time PCRs with *LAC2*-specific primers. The level of *LAC2* transcript in each sample was determined as an *n*-fold induction relative to the control condition (YNB [pH 7] with 2% glucose), using the comparative C_T method and using the *GPD* gene to normalize for RNA loading. Transcription of the *LAC2* gene was not significantly affected by oxidative stress (hydrogen peroxide or paraquat), nitrosative stress (pH 4) (plus $NaNO_2$), low pH, or exogenous copper. The only condition in which *LAC2* transcription was induced more than twofold was glucose deprivation (YNB plus 0.1% glucose). In H99 cells incubated in this glucose-poor medium for 2 h, *LAC2* was induced four- to fivefold compared to results for H99 cells incubated in a glucose-rich medium (Fig. 3B). These results are similar to those with the *LAC1* gene, which is similarly repressed by glucose (59).

The effects of other gene mutations on *LAC2* expression were also assessed by real-time PCR. In accordance with the microarray result, *LAC2* transcript levels are reduced in a *gpa1* mutant strain from wild-type levels (Fig. 3A). This further confirms that this signaling pathway transcriptionally regulates both *LAC1* and *LAC2*. In contrast, *LAC2* expression is increased in a *lac1* mutant strain compared to wild-type expression (Fig. 3A). However, as evidenced by the melanin defects of a *lac1* strain, this increase in *LAC2* message is not sufficient to fully restore wild-type levels of laccase activity to this mutant strain.

Disruption of the *LAC2* gene results in reduced melanin production. To determine the relative contribution of the Lac2 protein to melanin production, we used gene disruption and genetic epistasis approaches. The entire coding region of *LAC2*

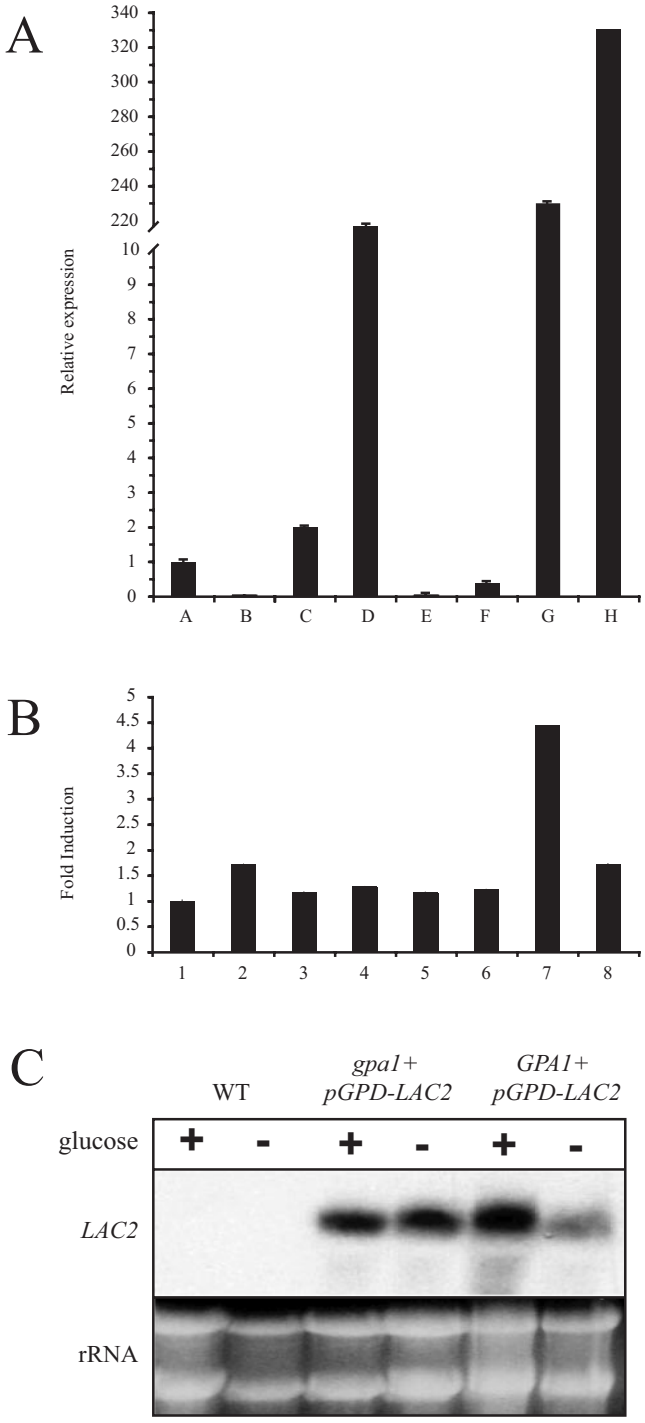


FIG. 3. Measuring LAC2 transcript using real-time PCR and Northern analysis. (A) Real-time PCR was used to determine the relative transcript levels of *LAC2* in each of the following strains after 1 h of glucose starvation: (A) wild-type (H99), (B) *lac2* mutant (RPC27), (C) *lac1* mutant (MDC16), (D) *lac2*+*pGPD-LAC2* mutant (QGC1), (E) *lac1 lac2* mutant (QGC9), (F) *gpa1* mutant (AAC1), (G) *gpa1*+*pGPD-LAC2* mutant (RPC18), and (H) wild type + *pGPD-LAC2* (RPC21). The results are demonstrated as expression relative to the wild-type strain H99. Each data point represents the average for triplicate samples with error bars indicated. (B) The wild-type strain H99 was incubated to mid-logarithmic phase in YPD medium and subsequently exposed to several different growth conditions for 1 h: (1) YNB (pH 7), (2) YNB (pH 4), (3) YNB (pH 7) plus 10 mM Na hydrogen peroxide, (6) YPD plus 10 mM paraquat, (7) YNB plus 0.01% glucose, and (8) YPD plus 100 μM copper sulfate. Total mRNA was isolated from these strains, treated with DNase-free RNase, and converted to cDNA. Relative *LAC2* transcript levels, indicated as *n*-fold induction compared to the baseline condition 1, were determined for each of the samples with real-time PCR. Each data point represents the average for triplicate samples with error bars indicated. (C) Northern analysis documenting *LAC2* overexpression. Total RNA was isolated from the wild-type (H99), *gpa1*+*pGPD-LAC2* (RPC18), and wild type + *pGPD-LAC2* (RPC21) strains after 1 h of incubation in a glucose-rich (+) or glucose-poor (−) medium and subjected to Northern analysis with the *LAC2* gene as a probe. The rRNA signal in the ethidium bromide-stained RNA gel is shown to demonstrate equal RNA loading.

was replaced with the dominant selectable marker encoding neomycin resistance (*neo*) (Fig. 4B). The resulting *lac2*::*neo* disruption allele was introduced into the serotype A wild-type strain H99 by biolistic transformation. In three independent transformants (RPC26, RPC27, and RPC28), the *LAC2* gene was precisely replaced by integration of the *lac2*::*neo* mutant allele, with no ectopic integrations. Two *lac1 lac2* double-mutant strains (RPC30 and QGC9) were created in a similar manner, using a *lac2* disruption construct to transform a serotype A *lac1* mutant strain. The *lac1* and *lac2* mutations were confirmed by PCR and Southern hybridization (Fig. 4A), and no *LAC2* transcript was detected in the *lac2* or *lac1 lac2* strains by real-time PCR (Fig. 3A). The phenotypes of three independent *lac2* mutants were identical, and we chose one of these strains (RPC27) as a representative *lac2* mutant for subsequent experiments. Similarly, the *lac1 lac2* double-mutant strains demonstrated identical phenotypes, and strain QGC9 was used in subsequent experiments as the *lac1 lac2* mutant strain.

Laccase activity in these strains was quantified by incubating the wild-type, *lac1* mutant, *lac2* mutant, and *lac1 lac2* double-mutant strains in YNB plus 0.1% glucose in the presence of three separate laccase substrates: niger seed extract, dopamine, and epinephrine. Laccase activity was quantified spectrophotometrically by the appearance of pigment in the culture supernatant. The wild-type strain exhibited 2.4-fold-greater laccase activity than the *lac2* mutant and fivefold greater laccase activity than the *lac1* mutant strain (Fig. 5A). This effect was identical whether Niger seed extract, dopamine, or epinephrine was used as the substrate for melanin production. Therefore, in three separate laccase assays using three different melanin substrates, we observed a consistent, impaired laccase activity due to a *lac2* mutation. With longer incubations, melanin formation by the wild-type and *lac2* mutant strain reached a saturating level. This observation is consistent with similar melanin levels apparent when these two strains were incubated on solid melanin-inducing media.

The *lac1* mutant strain demonstrated a more pronounced decrease in melanin activity than the *lac2* mutant strain. However, after prolonged incubation, this strain demonstrated notable pigment formation. This effect was not likely due to autooxidation of the melanin substrates, since cell-free controls demonstrated minimal melanin formation over the course of this experiment. Therefore, enzymes other than Lac1 are involved in *C. neoformans* melanin formation.

The *lac1 lac2* double mutant had the most profound defect

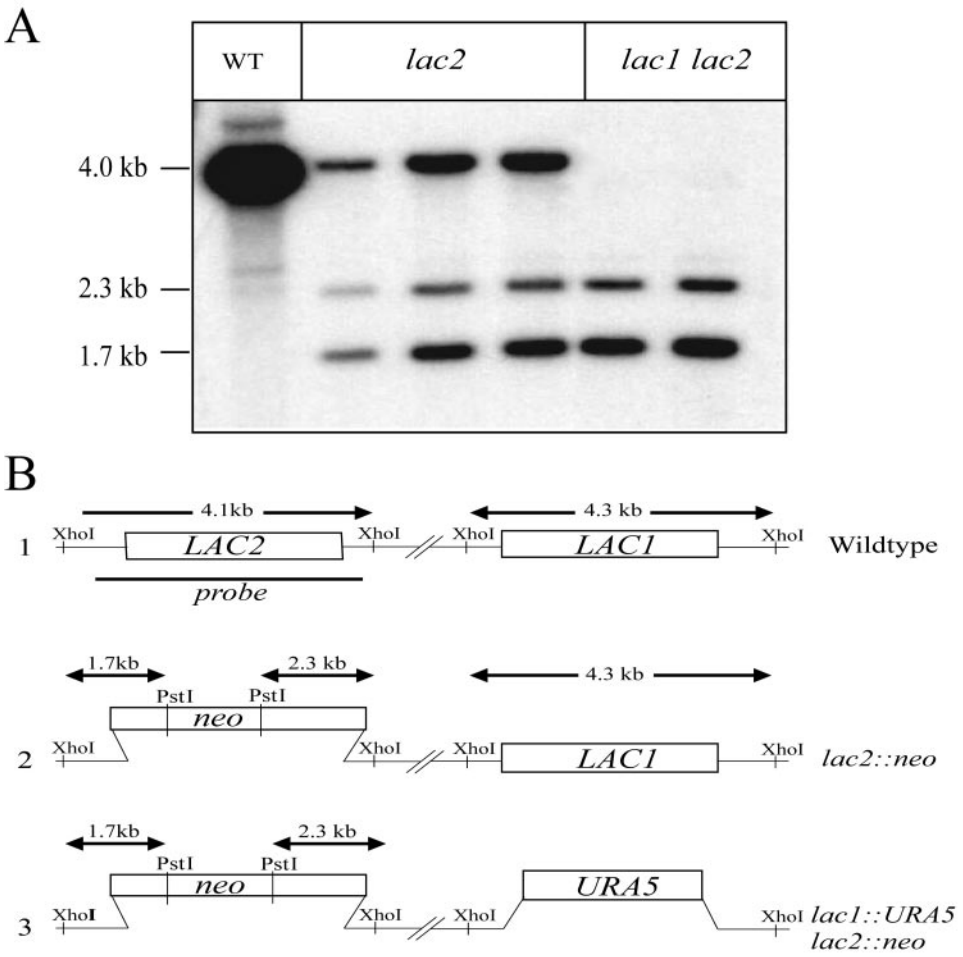


FIG. 4. Southern blot of *lac2* and *lac1 lac2* mutants. (A) Genomic DNA from the wild type (H99), *lac2* mutants (RPC26, RPC27, and RPC28), and *lac1 lac2* double mutants (RPC30 and QGC9) was digested with PstI and XhoI and examined by Southern analysis using the indicated region of the *LAC2* locus as a probe. (B) Restriction maps of the *LAC2* and *LAC1* loci.

in laccase activity, and little detectable pigment was formed in the presence of dopamine, epinephrine, or niger seed extract. Together, these findings support a model in which Lac1 and Lac2 play additive roles in *C. neoformans* melanin production.

Overexpression of the *LAC2* gene restores melanin to the *gpa1* and *lac1* mutants. To confirm that the *LAC2* sequence represents a functional gene involved in melanin production, we overexpressed this gene in four strain backgrounds. We cloned *LAC2* under the control of the constitutively active promoter of the glucose-6-phosphate dehydrogenase gene (*GPD*) and biolistically transformed this plasmid into the wild-type, *gpa1* mutant, *lac1* mutant, and *lac2* mutant strains. All transformants exhibited wild-type levels of growth on rich (YPD) and minimal (YNB) media, indicating that differences in growth rate or nutrient acquisition do not account for altered melanin production in these strains. Overexpression of *LAC2* in these strains was documented by real-time PCR and by Northern hybridization (Fig. 3A and C). The *LAC2* transcript was not detectable by this assay in the wild-type strain grown in the presence or absence of glucose. However, the *LAC2*-overexpressing strains demonstrated *LAC2* levels that were clearly detected by Northern analysis (Fig. 3C).

On melanin-inducing niger seed medium, the wild-type strain demonstrates vigorous melanin production that is repressed by higher glucose concentration (Fig. 5B). Overexpression of *LAC2* in the wild-type and *lac2* strains results in increased melanin production. This activity is especially evident on the medium containing 2% glucose, consistent with the constitutive activity of the *GPD* promoter. As previously noted (2), little melanin is produced by the *gpa1* mutant strain on either medium. *LAC2* overexpression completely suppresses the melanin-deficient phenotype of the *gpa1* mutant strain.

After 2 days of incubation on niger seed medium, no melanin was apparent in the *lac1* mutant strain. However, *LAC2* overexpression completely restored wild-type levels of melanin production in this strain. These findings indicate that the *LAC2* gene encodes a functional protein involved in melanin biosynthesis and that the Lac2 protein shares a redundant or overlapping function with Lac1. Also, the observation that the melanin defect of the *gpa1* mutant can be completely suppressed by *LAC2* overexpression further supports our hypothesis that the Gpa1-cAMP pathway controls *C. neoformans* melanin production at the level of transcription of laccase genes rather than at multiple steps in this pathway.

Substrate specificity of Lac2. *C. neoformans* cannot synthesize melanin de novo; rather, it requires the presence of diphe-

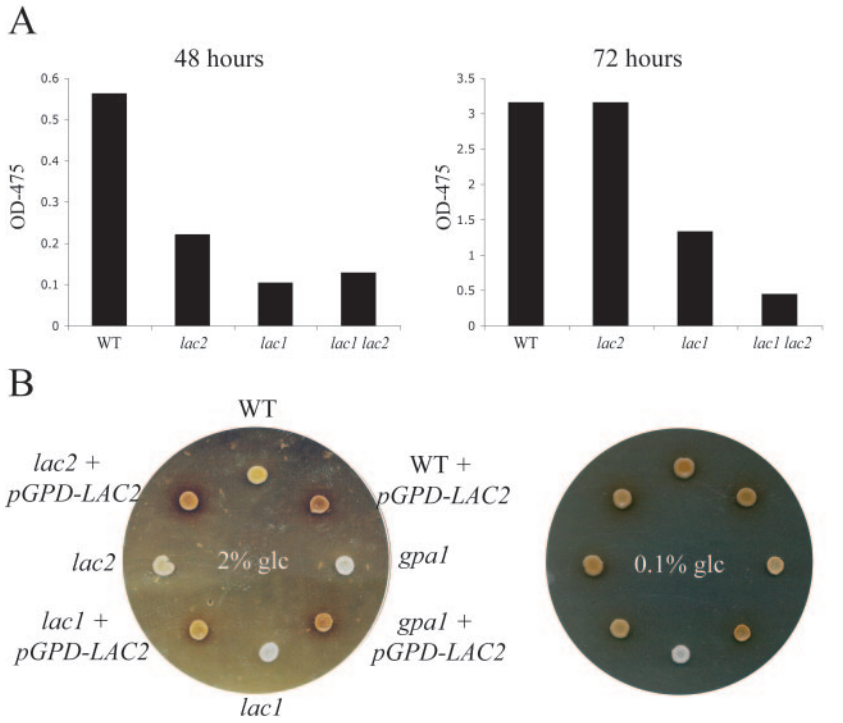


FIG. 5. Laccase activity. (A) The wild-type (H99), *lac2* mutant (RPC27), *lac1* mutant (MDC16), and *lac1 lac2* double mutant (QGC9) were incubated in YNB plus 0.1% glucose with 10 mM epinephrine. Laccase activity was quantified at 48 and 72 h by the appearance of pigment in the supernatant as assessed by measuring the absorbance at 475 nm. (B) The wild-type (H99), wild-type + *pGPD-LAC2* (RPC 21), *gpa1* (AAC1), *gpa1*+*pGPD-LAC2* (RPC18), *lac1* (MDC16), *lac1*+*pGPD-LAC2* (QGC1), *lac2* (RPC27), and *lac2*+*pGPD-LAC2* (QGC4) strains were incubated on niger seed medium with either 2 or 0.1% glucose for 72 h at 30°C. Melanin-producing strains make brown pigments on this medium.

nolic substrates, such as epinephrine, dopamine, L-DOPA, ABTS, or poorly characterized compounds present in niger seed extract. By selectively introducing different diphenols into the culture medium, we began to study the substrate specificity of laccases in different *C. neoformans* strains. We incubated the wild-type, *lac1* mutant, *lac2* mutant, *lac1 lac2* double mutant, and *lac1*+*LAC2* strains on media containing different substrates for melanin production (Fig. 6). *Guizotia abysinnica* seed

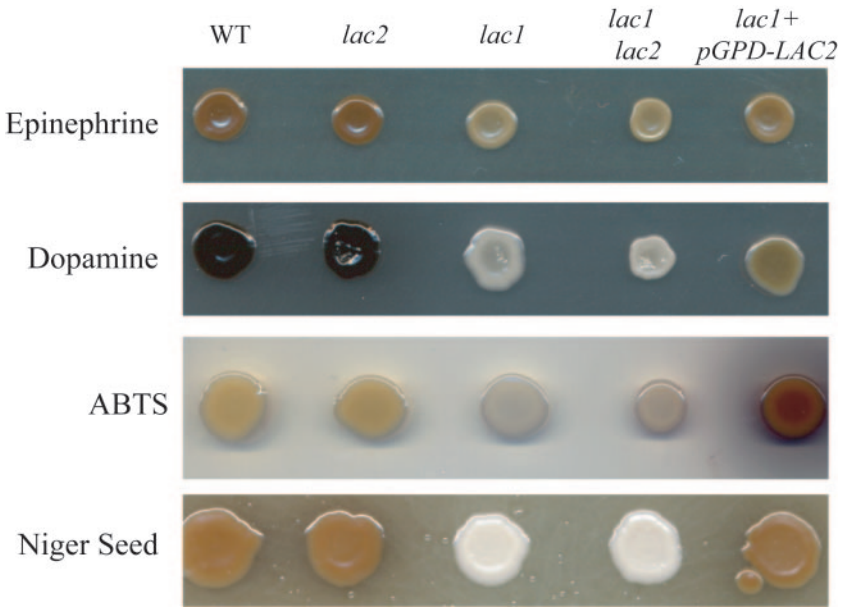


FIG. 6. Substrate utilization for melanin. The wild-type, *lac2* mutant (RPC27), *lac1* mutant (MDC16), *lac1 lac2* mutant (QGC9), and *lac1*+*pGPD-LAC2* strains (QGC1) were incubated for 72 h on YNB medium with 0.1% glucose containing one of the following substrates for melanin production: epinephrine, dopamine, ABTS, or niger seed extract.

(niger seed) extract contains a mixture of substrates used by *C. neoformans* to produce melanin. When incubated on a medium containing either niger seed extract or epinephrine, the *lac2* mutant strain makes melanin almost as efficiently as the wild-type strain, while the *lac1* mutant and the *lac1 lac2* double mutant do not produce visible melanin after 2 days of incubation. *LAC2* overexpression restored wild-type levels of melanin to the melanin-deficient *lac1* mutant strain (Fig. 5B and 6).

When these strains are incubated on medium containing ABTS, a different pattern of melanin production was observed. The *lac1* and *lac1 lac2* mutant strains remained melanin deficient compared with the isogenic wild-type and *lac2* mutant strains. However, the *LAC2* overexpression strain produced much more melanin than the wild type, resulting in a darker patch of cells than the wild type with a surrounding halo of green pigment (Fig. 6).

In contrast, strains overexpressing the *LAC2* gene do not make melanin efficiently when incubated on a medium containing dopamine as the substrate for melanin production (Fig. 6). Although melanin pigment is evident in the *lac1+LAC2* strain after 2 days of incubation on dopamine-containing medium, the degree of melanin production is much less than that of the wild-type strain.

A *lac1* mutation results in severely melanin-deficient strains on each of these media, and the Lac1 protein is therefore responsible for the majority of melanin production in vitro in the wild-type strain. Therefore, comparing wild-type and *LAC2* overexpression strains yields insight into the substrate specificities of the Lac1 and Lac2 proteins. Taken together, these results suggest that Lac1 and Lac2, when expressed at nearly equivalent levels, demonstrate similar substrate utilization for either epinephrine or the diphenols present in niger seed extract. The Lac2 protein is able to utilize ABTS as a melanin substrate more efficiently than the combination of laccases at the levels present in the wild-type strain. However, Lac2 poorly oxidizes dopamine for melanin biosynthesis. Such substrate specificity suggests that multiple laccases may allow *C. neoformans* to more efficiently produce melanin from different substrates encountered in a variety of environments.

***LAC2* and virulence of *C. neoformans* in an animal model of cryptococcosis.** The murine inhalation model of systemic *C. neoformans* infection was used to determine whether Lac2 is involved in virulence (Fig. 7) (3, 9). Female A/Jcr mice were intranasally inoculated with 10^5 *C. neoformans* cells, and animals were monitored for survival. As previously demonstrated, infection with the wild-type strain H99 results in a consistent lethal effect, with all mice succumbing to the infection between 23 and 27 days postinfection. Mice infected with a *lac2* mutant strain (RPC27) demonstrated no statistically significant difference in survival compared to the wild type ($P = 0.73$), suggesting that Lac2 is dispensable for *C. neoformans* virulence in this model system. Animals infected with a *lac1* mutant (MDC16) demonstrated prolonged survival compared either to the wild type ($P = 0.0002$) or to the *lac2* mutant ($P = 0.0002$), although all of the mice succumbed to a lethal infection by 35 days. Overexpression of the *LAC2* gene in the *lac1* mutant background, which restores melanin production, also suppressed the *lac1* mutant virulence defect ($P = 0.0004$).

In contrast, the *gpa1* mutant strain did not cause a lethal infection in any animal, even after 80 days of observation.

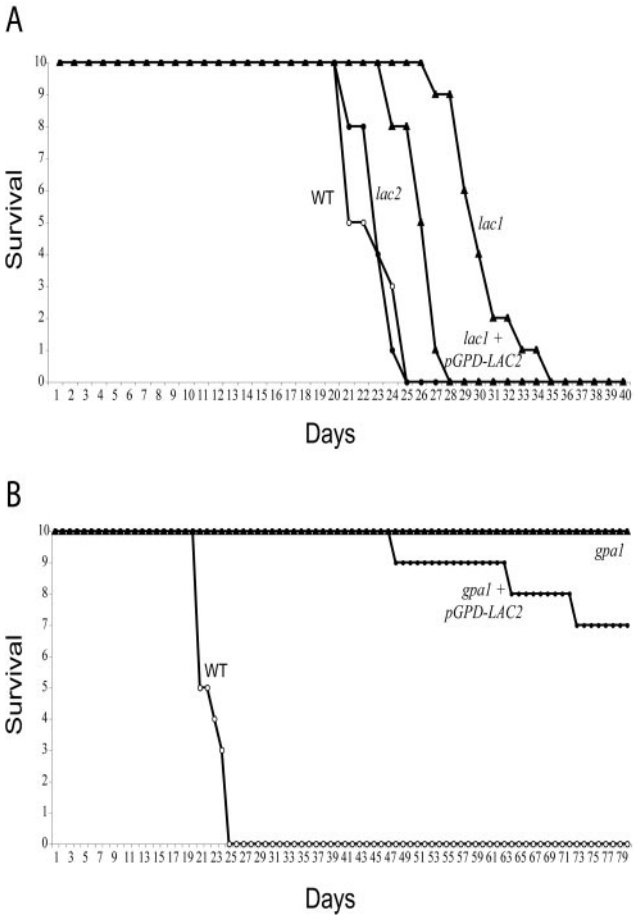


FIG. 7. LAC2 and the murine model of inhalational cryptococcosis. Female A/Jcr mice were intranasally inoculated with 10^5 cells of the following strains: wild type (H99), *lac2* mutant (RPC27), *lac1* mutant (MDC16), *lac1+pGPD-LAC2* strain (QGC1), *gpa1* mutant (AAC1), or *gpa1+pGPD-LAC2* strain (RPC18). The mice were monitored for clinical signs of cryptococcal infection and sacrificed at predetermined clinical end points that predict imminent mortality.

Previously, the *gpa1* virulence defect was complemented in the rabbit model of *C. neoformans* meningitis by reintroduction of the *GPA1* gene (2). *LAC2* overexpression completely restored melanin production in the *gpa1* mutant, but it was unable to restore virulence to this strain. This result is not unexpected, since the *gpa1* mutant strain also has a capsule defect, and capsule-deficient *C. neoformans* strains are attenuated for virulence (27). We observed three lethal infections in mice inoculated with the *gpa1+pGPD-LAC2* strain and none in the *gpa1* mutant, but the difference in these two virulence curves was not statistically significant. Therefore, constitutive Lac2 activity did not restore virulence to the hypocapsular *C. neoformans gpa1* mutant strain in this model system in contrast to its ability to complement the virulence defect of a *lac1* mutant.

DISCUSSION

In contrast to other microbial pathogens that are uniquely differentiated to survive within a specific host, many human fungal pathogens must be able to exist in the external environment as well as in an infected animal. In fact, many fungi that

are pathogens in humans are unlikely to encounter a mammalian host during their life cycle. This has led to the hypothesis that phenotypes offering a selective advantage for fungi within infected tissue might also play roles for survival in the environment. For example, melanized *C. neoformans* strains are more resistant to UV killing than identical strains in which melanin is not induced (57). Additionally, melanin-deficient strains are attenuated for survival when confronted with nonmammalian hosts, such as free-living amoebae and nematodes, which may be frequently encountered by fungi in the environment (35, 50). Encapsulated *C. neoformans* strains are also more resistant to killing by amoebae than nonencapsulated strains (50). Therefore, whether living within an infected host or in the external environment, the ability of microorganisms to coordinately regulate such apparently disparate phenotypes as laccase activity and capsule formation may be advantageous for survival.

We have previously demonstrated that the signal transduction pathway that controls *C. neoformans* cAMP metabolism regulates numerous cellular traits, including encapsulation, melanin formation, and mating (2, 3, 17, 20). Additionally, our aim is to define how one signaling pathway can link specific input signals to the induction of distinct phenotypic outputs. For example, nitrogen starvation is required for mating in *C. neoformans* and also for the transcriptional induction of *GPA1*. However, this environmental signal does not result in the induction of other cAMP-dependent phenotypes, such as melanin and capsule production. Therefore, the cAMP signal appears to be necessary, but not sufficient, for many of the phenotypes it regulates.

This model is perhaps most clearly illustrated in the case of *C. neoformans* mating. The ability of *C. neoformans* strains to mate efficiently requires both starvation and the presence of an appropriate mating partner. Recent studies have demonstrated that mutations either in the pheromone response pathway or in the cAMP pathway render the cell sterile (12, 13, 56). However, these two signaling pathways are quite distinct. It is likely that the cAMP pathway transduces nutrient deprivation signals required for mating, and the mitogen-activated protein kinase pheromone response pathway signals the presence of a mating partner. Similarly, melanin and capsule formation may also require multiple environmental signals for induction, and the cAMP pathway likely provides only one of these important inputs.

Numerous proteins are involved in capsule synthesis in *C. neoformans*. In fact, several capsule genes have been identified whose encoded protein has a yet-unidentified function (5–8, 16, 22). However, a coordinated regulation of proteins involved in capsule synthesis and assembly would likely benefit the cell. Our observation that the Gα protein Gpa1 regulates the transcription of multiple capsule genes begins to define a mechanism by which this signaling pathway allows synchronized capsule gene expression. Initial analysis of the promoter regions of the coregulated capsule genes failed to demonstrate large regions of identity. However, more-detailed promoter evaluations will likely be necessary to elucidate *cis*- and *trans*-acting regulatory elements that control capsule gene expression in response to a cAMP signal.

Fungal melanins are also required for survival of several fungal species within mammalian hosts. In human-pathogenic fungi, such as *Aspergillus fumigatus*, *Paracoccidioides brasiliensis*, *Sporothrix schenckii*, *W. dermatitidis*, and *C. neoformans*, melanins play a protective role against effectors of cellular immunity and possibly a mechanical role in pathogenesis (28). Therefore, the synthesis of melanin has large implications from the mechanisms of fungal virulence to treatment of infection in the host.

In these studies we identified a second *C. neoformans* laccase-encoding gene, *LAC2*, whose product is involved in the melanin biosynthetic pathway. Laccases (benzendiol:oxygen oxidoreductases) are blue, multicopper-containing enzymes that catalyze oxidation of a range of aromatic compounds in different fungal species. Laccases are important catalysts in the oxidative steps of melanin formation. In wood-rotting fungi, laccases also play a prominent role in lignin degradation, allowing parasitism of woody plants (30). Aside from this catalytic role, they are also involved in other physiological processes. For example, fruiting body formation in ascomycetes is dependent on laccases (21, 51). Extracellular laccase-like, multicopper oxidases have also been shown to have ferroxidase activity in the basidiomycete *Phanerochaete chrysosporium* (29). Laccase activity may also participate in combating oxidative stress under normal quiescent conditions (18).

Analysis of the genomes of the serotype A strain H99, the serotype D strain JEC21, and the serotype B strain WM276 indicates that *LAC1* and *LAC2* are the result of an ancient duplication event which occurred prior to the differentiation of the *C. neoformans* varieties. In all of these strains of divergent varieties, the two laccase genes are adjacent to one another and display the same gene orientation. Additionally, phylogenetic analysis of the two laccase genes clearly indicates that all of the *LAC1* homologs are more closely related to one another than to other laccase genes within the same strain. Similarly, the *LAC2* genes in these three strains are more homologous to each other than to *LAC1*. Further studies defining the different functions of these laccases will illuminate why *C. neoformans* maintained two such highly similar genes as the three varieties differentiated and came to occupy different ecological niches.

Several aspects of *C. neoformans* laccase function have already been described. For example, the Lac1 enzyme is well characterized for its role in the synthesis of melanin (60). However, the presence of other, similar enzymes in this organism was implied by several observations. First, although *lac1* mutant strains have a striking melanin defect, these strains eventually produce melanin pigments. This observation may be explained by autooxidation of melanin precursors or the presence of alternative phenoloxidases capable of catalyzing this step in melanin formation. Also, in other basidiomycetes, multiple laccase genes have been identified. For example, eight nonallelic laccases have been defined in *Coprinus cinereus* (21). Several of these genes are certain to encode oxidases involved in iron metabolism. However, the presence of a gene family with eight apparent members raises interesting questions about the importance of this group of enzymes. Do all of these enzymes perform completely separate and distinct functions, or is there some degree of functional redundancy present among fungal laccases?

In our studies, we demonstrate that the Lac2 protein, though expressed at low levels under the conditions tested thus far in vitro, serves a redundant function in melanin formation with

Lac1. When overexpressed, the *LAC2* gene suppressed the melanin defect of the *lac1* mutant strain. This suggests some degree of functional redundancy, rather than completely distinct functions, among these fungal laccases.

Because fungi are likely to encounter a variety of phenolic compounds in the environment and in the host, the ability to utilize many different compounds as precursors for melanin would offer survival advantages under these very different conditions. Others have suggested that multiple laccases would offer such functional elasticity to pigmented fungi, rather than requiring each laccase to oxidize various substrates (21). We demonstrated that a *C. neoformans* strain overexpressing the *LAC2* gene is able to use several catecholamines with differing efficiencies for melanin production. Additionally, this strain displays different substrate utilization patterns compared with a wild-type strain in which the Lac1 protein is the predominant laccase. This result supports the hypothesis that the presence of multiple laccases provides fungal cells with a broader array of potential substrate utilization patterns.

Four diphenols are present in human brain tissue: L-DOPA, norepinephrine, epinephrine, and dopamine. *C. neoformans* likely uses these substrates in vivo for melanin biosynthesis, since this yeast has demonstrated production of melanin in animal models of cryptococcosis and in human infections (37, 38). It will be interesting to determine which laccases are functioning in vivo to use these catecholamines for melanin biosynthesis.

More evidence of differential laccase function within this family is suggested by the different expression patterns of the genes. By Northern blot and quantitative PCR analysis, the level of *LAC2* transcript is significantly lower than that of *LAC1*. Likewise, laccases in *C. cinereus* are shown to have wide variation in expression patterns. Three of the eight isolated laccases in this organism are differentially regulated by nutrients and the presence of metallic and aromatic inducers (21). In other fungi, laccases are induced by such varied signals as temperature, osmotic pressure, and plant secondary metabolites (40, 48). Defining the inducing conditions for cAMP-regulated genes will help to determine the specific upstream signals that activate the cAMP cascade in *C. neoformans*.

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